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Determining the Mechanism of Six1-Induced TRAIL Resistance and Its Role in Breast Cancer Metastasis

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14. ABSTRACT

Breast cancer is the most common cancer in women and the second deadliest. There is a great need to finding new targeted therapies and to improve the efficacy of existing therapies. The TNF Related Apoptosis Inducing Ligand (TRAIL) pathway is part of the body's natural tumor surveillance program, preventing formation of tumors and metastasis while sparing normal cells. The TRAIL pathway has been exploited in clinical trials but resistance to TRAIL is common, limiting the efficacy of therapy. The mechanisms underlying TRAIL resistance are largely unknown and there is of yet not a good way to screen for TRAIL sensitivity. We have found that the gene Six1, which is overexpressed in over half of all breast cancers and in as much as 90% of metastatic lesions, confers resistance to TRAIL. In addition, by screening a genome wide shRNA library we have identified 4 novel TRAIL resistance gene including the solute carrier family 26 (sulfate transporter), member 2 (SLC26A2). The role of these genes in TRAIL resistance and metastatic spread are being investigated, with the ultimate aim of identifying TRAIL resistance and circumventing it through targeted combination therapies.

15. SUBJECT TERMS
Six1, TRAIL resistance, breast cancer, genome-wide shRNA library screen

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Annual Report Lina Dimberg Department of Defense

INTRODUCTION

Breast cancer remains the second deadliest cancer in women. The search for a therapy that kills cancer cells efficiently while sparing normal cells is highly warranted. The TNF Related Apoptosis Inducing Ligand (TRAIL) pathway is part of the body's natural defense against tumor and as such provides a very attractive target of therapeutic intervention. However, in the very nature of tumor formation lies the ability to evolve ways to counteract such growth constraints. Indeed, many tumors are inherently resistant to TRAIL or gain resistance during the course of TRAIL treatment. In order to exploit the TRAIL pathway, it is of utmost importance that we learn to understand the underlying mechanisms of TRAIL resistance and sensitivity. Only then can we predict which patients are likely to benefit from treatment and be able to counteract resistance as it arises.

We have identified Six1 as a mediator of TRAIL resistance. This is an important discovery since this gene is overexpressed in 90% of metastatic lesions and is associated with worsened clinical outcome in breast cancer patients (1) (2) (2) . We have now continued to screen for additional novel genes, related or unrelated to Six1, that influence resistance to TRAIL. Our ultimate goal is to find biomarkers of TRAIL resistance in breast cancer and also to find druggable pathways that, when targeted in combination with TRAIL treatment, enhance tumor killing and reverse resistance in breast cancer patients.

BODY

Task1: To develop cell line systems where Six1 expression can be regulated by inducible overexpression and/or knockdown and to characterize TRAIL resistance in these cells (Year 1)

A. Six1 vector construction and verification (months 1-3)
B. Inducible knockdown of Six1 in 21PT cells (months 3-5)
C. Inducible overexpression of Six1 in MCF7 and MCF12-A cells (months 5-7)
D. Knockdown of Six1 in 4T1 cells (months 7-9)

As outlined in my previous annual report, we decided to primarily use a BJAB lymphoma cell line as a model system for TRAIL sensitivity. In addition to the parental cell line, one of these cell lines have a stable overexpression of Six1 and one, LexR, has been made resistant to TRAIL through repeated long term exposure to increasing concentrations of agonistic TRAIL antibody (lexatumumab). We reasoned that while expression of Six1 clearly leads to a more TRAIL resistant phenotype, the downstream mechanism could not be found within the TRAIL pathway, the exception being Bid which was insufficiently cleaved (activated) in the Six1-cells as compared to the control cells. It is plausible that important TRAIL resistance mechanisms, that may or may not be downstream of Six1, are novel rather than being part of already established apoptosis pathways . To explore this we performed the shRNA screen described under Task 2. Again, because of technical

reasons, the cell lines that we used were lymphoma rather than breast cancer cell lines. When assaying a panel of breast cancer cell lines for TRAIL resistance as described in the previous annual report, we found that the MDAMB231 cell line was sensitive to TRAIL to roughly the same extent as the sensitivity found in parental BJAB cells. In order to be able to corroborate findings from the LexR screen in a breast cancer system, we made cell lines, MDAMB231resistant and MDAMB231sensitive, which were or were not exposed to increasing concentrations of TRAIL during a prolonged period of time.

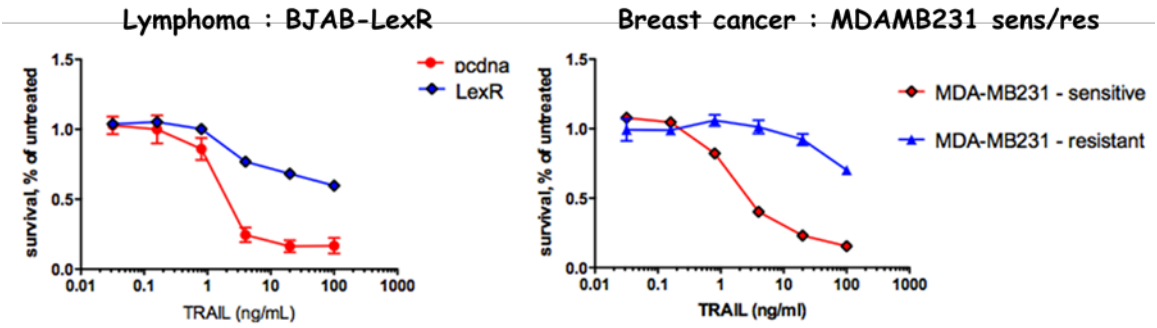


Fig1: Lymphoma cell lines BJAB-LexR (TRAIL resistant) and BJAB-pcDNA (control cells, TRAIL sensitive) (left panel) and breast cancer cell lines MDA-MB-231 sensitive and resistant (right panel) were treated with varying concentrations of recombinant TRAIL for 24 hours. Survival as a percentage of untreated cells was determined by CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay .

As shown in dose-response MTS assays (fig 1,) this approach successfully resulted in MDAMB231 cell lines that, in analogy to LexR, were highly resistant to TRAIL as compared to their parental counterparts. These cells are currently being used to corroborate the results from the unbiased shRNA screen as described under Task 2.

Task 2

A. Mining the microarray data of RNA from CAT transfected vs. Six1 transfected MCF12A cells and MCF7 cells previously generated in our laboratory for candidate genes that are upregulated or downregulated by Six1 and that may be involved in TRAIL-induced apoptosis, (month 13)

As previously reported, this approach was abandoned since we could not find evidence for Six-mediated TRAIL resistance in MCF7 cells and since the MCF7 cell lines are deficient in caspase 3. Instead, we performed new miocroarrays, both with the BJAB LexR cell line and its sensitive parental counterpart and with the MDAMB231 RES/SEN cells. We included both untreated cells, to see how basal gene expression was changed by acquired TRAIL resistance, and TRAIL treated cells, to see whether different changes in gene expression in sensitive versus resistant cells could explain the mechanisms of acquired TRAIL resistance. We are currently using this expression analysis data to corroborate findings from the shRNA library

screening described under Task2B, both in terms of specific genes and in terms of finding downstream effectors of identified candidate targets. Also, we want to find common, preferably druggable pathways in both of these systems that could synergize with TRAIL. One such pathway, the Ras/MAPK pathway, exhibited numerous hits in terms of being upregulated in both TRAIL treated, TRAIL resistant MDA-MB-231 breast cancer cells and in TRAIL-treated TRAIL resistant BJAB LexR cells. In addition, several components of this pathway were also identified as resistance genes in the shRNA screen.

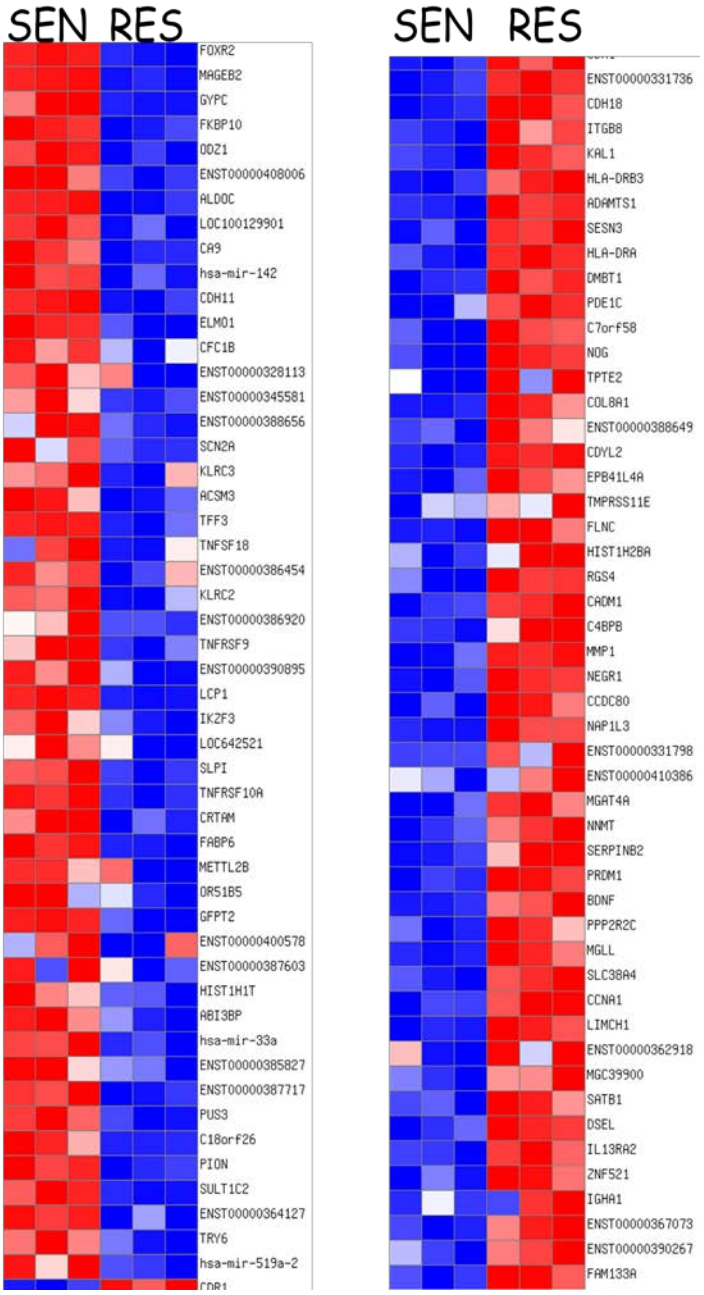


Fig 2: Basal gene expression in TRAIL sensitive versus TRAIL resistant MDA-MB-231 cells in a Affymetrix Human Gene 1.0 ST Array. Heat map indicates upregulation in red and downregulation in blue

B. Performing and analyzing the shRNA library screen in the cell lines generated under Task1. We will determine which shRNAs reverse the Six1-induced resistance to TRAIL induced apoptosis in Six1-overexpressing cells, i.e.which shRNAs that are under-represented in Six1-overexpressing cells that survive TRAIL treatment vs untreated cells. These shRNAs should target genes that mediate TRAIL resistance induced by Six1. Conversely, shRNAs that induce resistance to TRAIL in cells where Six1 is knocked out would be over-represented in the surviving TRAIL-treated population vs in untreated cells and should be negative regulators of TRAIL resistance (month 14-17)

As described in the previous annual report, we performed shRNA screens on Six1-overexpressing cells and also on the TRAIL resistant LexR cells. These initial screens gave rise to lists of candidates, 239 and 580 genes, respectively, from which we selected a subset of genes, 101 and 212, respectively. Plasmids encoding shRNAs targeting these genes were pooled, creating a secondary shRNA sub-library. Again, lists of putative resistance genes were generated and the hits were then examined one by one using individual shRNAs from the UC Denver Functional Genomics Core.

C. Corroborating Six1 upregulation of DcR1 + other candidates by Northern, RT-PCR, Western blot, and/or flow cytometry (month 17-21)

This aim was in part completed in the previous period where we analyzed regulation of several TRAIL-related proteins including TRAIL receptor protein expression, IAP proteins, Bcl2 proteins, FLIP and caspase 8. Of these apoptosis proteins, we found evidence for impaired Bid-cleavage only in Six1.

D. Designing shRNA + acquiring cDNA for vectors, constructing vectors. These vectors will be used for transient knockdown/overexpression studies (months 21-25)
E. Transient knockdown/ overexpression studies to determine impact of candidate genes on TRAIL sensitivity (months 26-30)

After evaluating the hits one by one using individual shRNAs , we found that the most promising hits are all in the LexR system. Part of the reason for this is some technical difficulties: the vector expressing Six1 in the Six1-BJABs already contains the puromycin resistance gene and therefore we are unable to select for individual shRNAs. Since knocking out our putative resistance gene reverses the resistance originally provided by Six1 there is a strong selection against maintaining such an shRNA. Because of this we have chosen to initially focus more on our general models of acquired TRAIL resistance at the time being, and to corroborate the Six1 shRNA findings in a breast cancer system such as the aforementioned BT549 system, knocking down Six1 using shRNA vectors that do not confer puromycin resistance. Although we initially set out to primarily focus on Six1-mediated resistance to TRAIL, we believe that a more generalized approach is likely to shed

light on novel TRAIL resistance mechanisms that could be of importance in Six1-overexpressing cells as well.

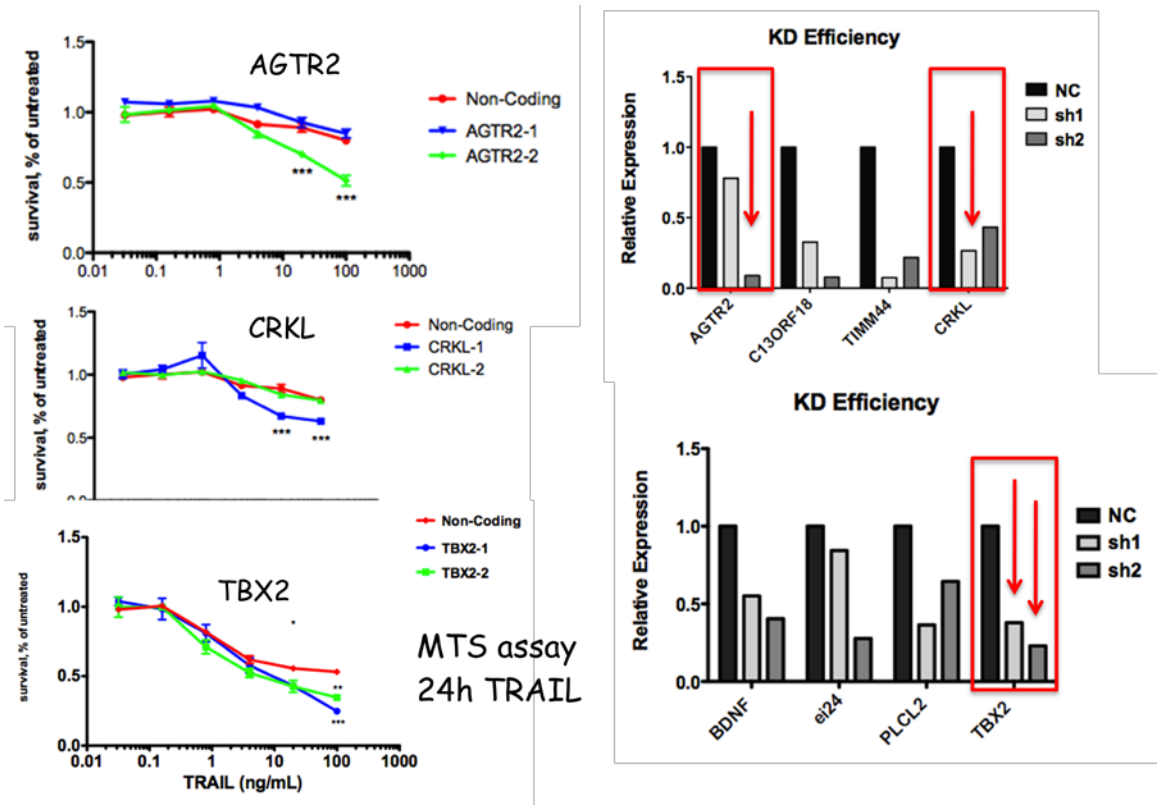


Fig3: Lymphoma cell lines BJAB-LexR (TRAIL resistant) were lentivirally transduced with either control, non targeting shRNA vector (Non-Coding) or with either of two different individual shRNAs targeting the indicated genes AGTR2, CRKL and TBX2, respectively, The cells were treated with varying concentrations of recombinant TRAIL for 24 hours. Survival as a percentage of untreated cells was determined by CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (right panel) . Knockdown was determined by realt time quantitative PCR using primers specific to the indicated genes. The expression is relative to the expression of GAPDH and was normalized to non-coding shRNA vector expression.

Three of the most prominent targets were Angiotensin II Receptor 2 (AGTR2), Crk-like protein (CRKL) and T-Box Transcription Factor 2 (TBX2). When these genes were knocked down, using two different individual shRNAs, resistance to TRAIL was reduced as compared to non-targeting shRNA control (fig3, left panel). With the exception of CRKL, the degree of enhanced sensitivity correlated well with the degree of knockdown (fig3, right panel). It must be noted that there may be a threshold that needs to be reached before knockdown can give any effect. AGTR2 is highly expressed in fetal development at mediates programmed cell death (3). CRKL activates Ras and Jun and has oncogenic potential (4). The connection to Ras is especially interesting since our microarray data shows that components of the Ras/MAPK pathway are upregulated in TRAIL-resistant lymphoma cells and breast

cancer cells during TRAIL treatment. TBX2 is involved in transcriptional regulation of p53 but also in the Wnt /beta catenin pathway (5) which previously came up in our shRNA screen as an important pathway in Six1-mediated resistance. Importantly, none of these targets have to our knowledge been directly linked to TRAIL-induced apoptosis previously. We will confirm these results in clonal isolates of BJAB-LexR cells and also in our MDA-MB-231 breast cancer cells.

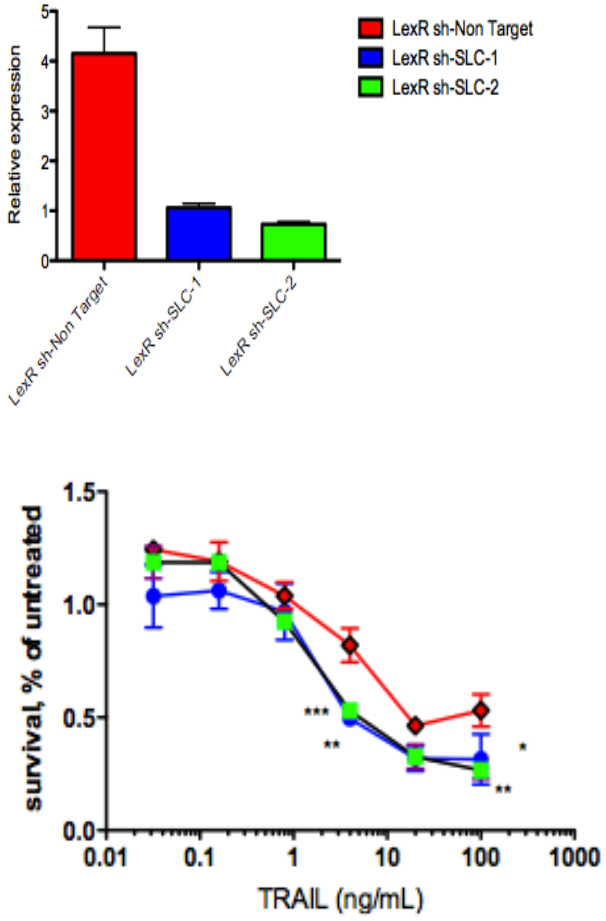


Fig4: Lymphoma cell lines BJAB-LexR (TRAIL resistant) were lentivirally transduced with either control, non targeting shRNA vector (Non Target) or with either of two different individual shRNAs targeting SLC26A2. The cells were treated with varying concentrations of recombinant TRAIL for 24 hours. Survival as a percentage of untreated cells was determined by CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (lower panel) . Knockdown was determined by real time quantitative PCR using primers specific to SLC26A2. The expression is relative to the expression of GAPDH (upper panel).

Another target that was verified was solute carrier family 26 (sulfate transporter), member 2 (SLC26A2). In pooled populations of shRNA-expressing cells TRAIL resistance was consistently reversed by both individual shRNA constructs (fig 4). Next, we examined whether the reduction in TRAIL resistance was accompanied by an increase in apoptosis. In an Annexin V/PI assay of cells treated with 100 ng/ml TRAIL for 2 hours we found that the proportions of apoptotic cells (defined as Annxin V+/PI - cells) were indeed increased in the SLC26A2 knockdown cells as compared to in non-targeting controls. (Table 1)

	Live cells	Apoptotic cells
Lex NT	68 %	5 %
Lex NT + TRAIL	39 %	30%
Lex sh-slc-1	74%	5%
Lex sh-slc-1 + TRAIL	22%	58%
Lex sh-slc-2	62%	9%
Lex sh-slc-2 + TRAIL	10%	75%

Table 1: Lymphoma cell lines BJAB-LexR (TRAIL resistant) were lentivirally transduced with either control, non targeting shRNA vector (NT) or with either of two different individual shRNAs targeting SLC26A2 (sh-slc-1 and sh-slc-2) and were either left untreated or were treated with 100 ng/ml TRAIL for 2h. Live cells and apoptotic cells were identified using an AnnexinV/PI assay.

The selective disadvantage of carrying an shRNA against a resistance gene makes it difficult to maintain a sufficient degree of knockdown in a pooled population. Because of this, we next established clonal isolates of both individual SLC26A2 shRNA constructs by single cell flow cytometry sorting. This approach yielded clones with a higher degree of knockdown and with a much more prominent effect on TRAIL sensitivity than in the pooled population (fig 5).

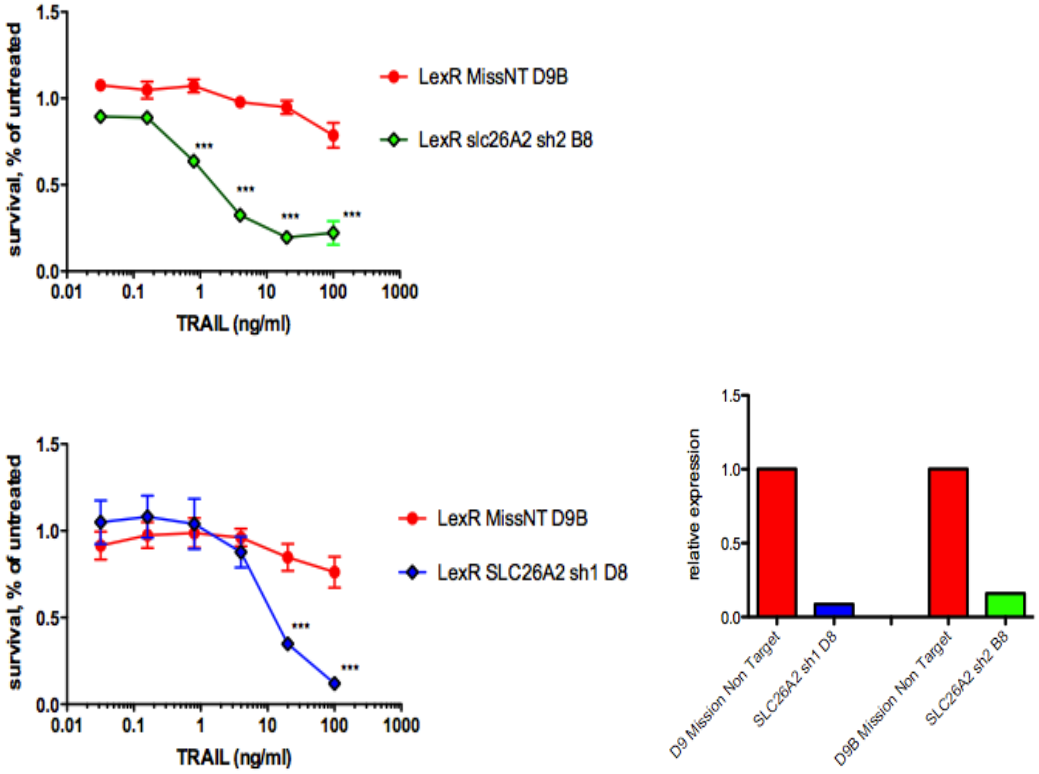


Fig5: Lymphoma cell lines BJAB-LexR (TRAIL resistant) were lentivirally transduced with either control, non targeting shRNA vector (Miss NT) or with either of two different individual shRNAs targeting SLC26A2. The cells were single sorted into 96 well plates using flow cytometry. Clonal isolates were selected. The cells were treated with varying concentrations of recombinant TRAIL for 24 hours. Survival as a percentage of untreated cells was determined by CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (left panel) . Knockdown was determined by real time quantitative PCR using primers specific to SLC26A2. The expression is relative to the expression of GAPDH (right panel).

SLC26A2 is a transmembrane glycoprotein that is involved in the sulfation of proteoglycans. It is critical for cartilage formation and mutation of this gene is implicated in the pathogenesis of human chondrodysplasias (reviewed in (6). As of yet, there is no evidence for any involvement of SLC26A2 in TRAIL-induced apoptosis. There is, however, examples of an involvement of proteoglycans in apoptosis regulation. Notably, downregulation of the heparan sulfate proteoglycan syndecan-1 is associated with enhanced sensitivity to TRAIL as well as an upregulation of GALNT3, (7) a gene belonging to the same family as the know TRAIL sensitization biomarker GALNT14 (8). It is plausible that a protein that could affect proteoglycan sulfation may influence TRAIL sensitivity by as yet unknown pathways. We will mine our microarray data for proteoglycans that may be important in TRAIL resistance.

We were very encouraged to see in preliminary experiments that with sufficient knockdown, SLC26A2 seems to reverse resistance in the MDAMB231 breast cancer system as well as (fig 6).

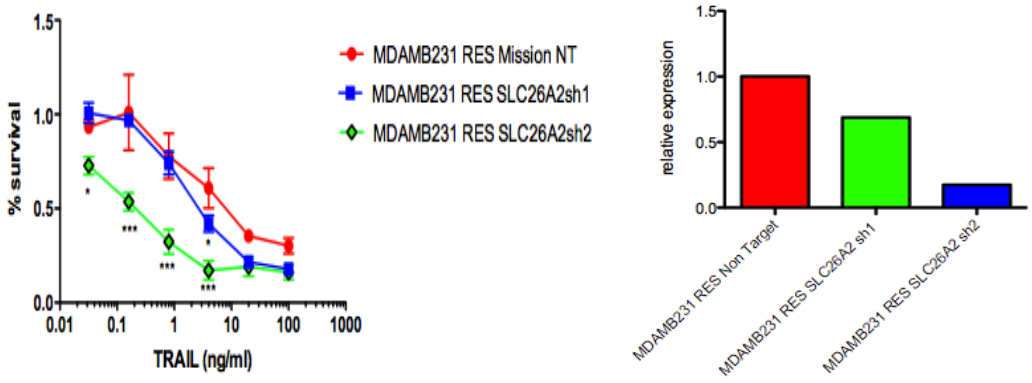
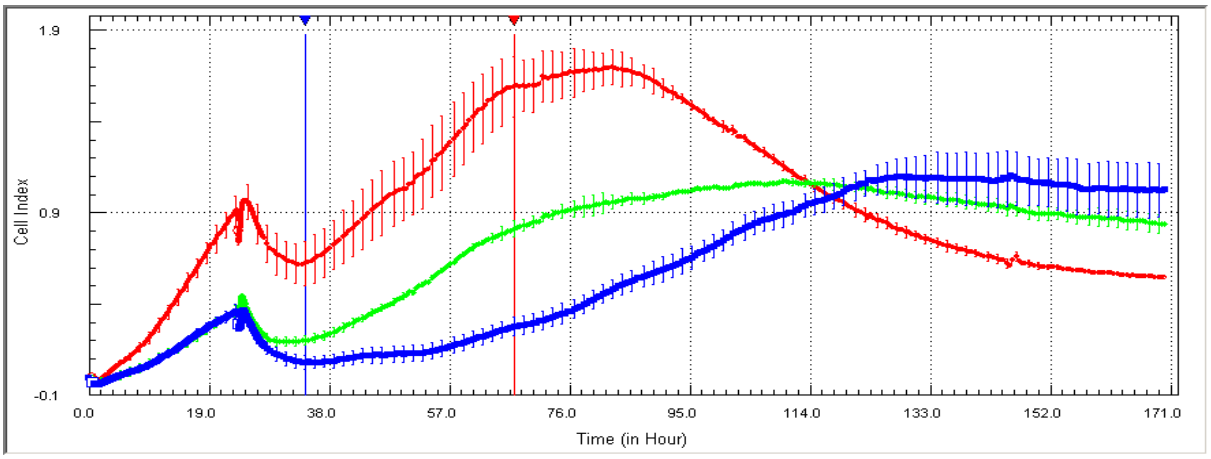


Fig6: Breast cancer cell lines MDA-MB-231, sensitive and resistant to TRAIL, were lentivirally transduced with either control, non targeting shRNA vector (Miss NT) or with either of two different individual shRNAs targeting SLC26A2. The cells were single sorted into 96 well plates using flow cytometry. Clonal isolates were selected. The cells were treated with varying concentrations of recombinant TRAIL for 24 hours. Survival as a percentage of untreated cells was determined by CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (left panel) . Knockdown was determined by real time quantitative PCR using primers specific to SLC26A2. The expression is relative to the expression of GAPDH (right panel).

In addition, using Xcelligence which quantifies cell attachment as a measure of cell viability and growth in a label-free manner in real time, we found a decreased growth/viability in the cells where slc26A2 was efficiently knocked down (fig7). In order to verify these results with two separate efficient knockdowns we will establish clonal isolates of SLC26A2 in MDAMB231RES



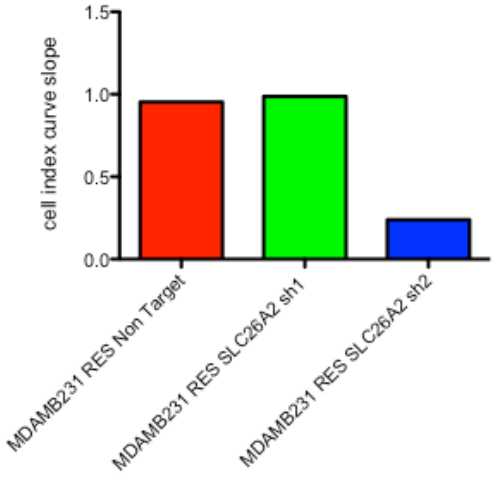


Fig 7: The upper panel shows the cell index corresponding to impedance, a measure of the degree of attachment of cells, plotted for MDAMB231 RES cells transduced with non targeting shRNA (red slope) and two different shRNAs against SLC26A2 designated sh1 (green slope) and sh2 (blue slope). TRAIL 4 ng/ml was added at 24 hours. The slope of the curve, left panel, is calculated between approximately 10 hours and 43 hours past start of treatment and has been normalized to the growth/survival rate in untreated cells

One caveat with knockdown with shRNAs is the risk of off target effects due to insertion sites. Reversal of TRAIL resistance in several clones of two separate individual shRNAs in the BJAB LexR system and a good correlation with the level of knockdown are both strong indicators that SLC26A2 is, in fact a resistance gene. However, to prove this we need to rescue SLC26A2 expression using a cDNA vector lacking the 3'UTR in a cell line clone expressing SLC26A2sh1, an shRNA that targets the 3'UTR. For this purpose, we have obtained an SLC26A2 vector from Dr Antonio Rossi which we will clone into an MSCV-IRES-GFP vector to create a MSCV-SLC26A2-IRES-GFP plasmid, enabling selection of transfected cells by flow cytometry. If reintroduction of the putative resistance gene restores TRAIL resistance in SLC26A2sh1-transduced cells we can be certain that SLC26A2 is indeed a TRAIL resistance gene. We will perform rescue experiments for every validated resistance gene.

Task 3: To test whether Six1-induced metastasis involves resistance to TRAIL-induced apoptosis

A. Transfect cell lines MCF-7 and 4T1 with candidate gene cDNA or shRNA (month 30-32)
B. Inject MCF7 and 4T1 cell lines into nude mice and immunecompetent mice, respectively. Evaluate metastasis. (month 32-36).

We wish to modify this aim to include increased metastasis as a result of general acquired TRAIL resistance as well. In addition, we will not use MCF7 in this system because it is unsuitable due to defect caspase activation. Instead we will use the MDA-MB-231 RES/SEN cells. We will first determine whether cells that are resistant to TRAIL will metastasize more than cells that are not by injecting MDA-MB-231 SEN and and MDA-MB-231 RES into nude mice and evaluate the number of metastases. Then, we will inject 1) clonal isolates of MDA-MB-231 RES where identified TRAIL resistance genes have been knocked out down by an shRNA targeting the 3'UTR and 2) clonal isolates of MDA-MB-231 RES where identified TRAIL resistance genes have been knocked down by an shRNA targeting the 3'UTR and then rescued by transfection with plasmid encoding cDNA of the resistance gene lacking the 3'UTR and evaluate the number of metastases in nude mice. We will then repeat the experiment using 4T1 cells so that immunocompetent mice can be used.
The new task description would be as follows:

Task3: To test whether acquired TRAIL resistance will increase metatasis of breast cancer cells and if so, whether knock-down of an indentified TRAIL resistance gene would prevent increased metastasis

A. Inject MDA-MB231-RES and MDA-MB231-SEN into nude mice and immunocompetent mice, evaluate metastasis(month 30-32)
B. Transduce cell lines MDA-MB231-RES cells and 4T1 cells with candidate gene shRNA and cDNA (month 33)
C. Inject transduced MDA-MB231-RES cells and 4T1 cells into nude mice and immunecompetent mice, respectively. Evaluate metastasis. (month 34-36).

We have not yet begun this task as it is dependent on first performing a thorough validation of genes that mediate TRAIL resistance.

KEY RESEARCH ACCOMPLISHMENTS

- Identification of a list of putative novel TRAIL resistance genes including SLC26A2, AGTR2, CRKL and TBX2
- Establishment of a breast cancer cell line system, MDA-MB-231 SEN and MDA-MB-231 RES, in which to study acquired resistance to TRAIL-induced apoptosis

- Performance of microarray analyses for both the BJAB LexR/pcDNA system and the MDA-MB-231 RES/SEN system identifying, among others, the Ras/MAPK pathway as playing a role in resistance to TRAIL-induced apoptosis

REPORTABLE OUTCOMES

- Oral presentation at the Department of Pharmacology Annual Retreat, October 2012
- Poster presentation at the AACR meeting Molecular Targets and Cancer Therapeutics, Dublin, Ireland, November 2012
- Published review article for Oncogene May 2012:
  Dimberg L Y, Andersson A, Behbakht K, Thorburn A, Camidge R, Ford HL On the TRAIL to successful cancer therapy? Predicting and counteracting resistance against TRAIL-based therapeutics

CONCLUSIONS

We set out to study the mechanism of Six1 induced resistance to TRAIL induced apoptosis in breast cancer and its role in metastasis. Although we are still interested in mechanisms related to Six1 expression, we have broadened our studies to other novel mechanisms of acquired resistance to TRAIL as well that may or may not converge with Six1 expression. Using that approach, we have found several novel TRAIL resistance gene candidates., SLC26A2, CRKL, AGTR2 and TBX2, that we are currently validating in rescue experiments. In particular, knocking down the gene SLC26A2 using either of two separate individual shRNA constructs enhances TRAIL sensitivity dramatically. In order to validate these results in breast cancer, we have developed MDA-MB-231 breast cancer cell sublines that mimic the BJAB system of TRAIL resistant – TRAIL sensitive cells. SLC26A2 seems to mediate resistance to TRAIL in these cells as well. We have also performed microarrays with the MDA-MB-231 cells in parallel with the BJAB cells to find common pathways affected during acquired TRAIL resistance. We found that the Ras/MAPK pathway is one common pathway that is induced during TRAIL treatment in resistant cell lines and that this pathway also contains several of the putative TRAIL resistance genes identified in the shRNA screen, including CRKL. This may be a feasible pathway to target therapeutically in combination with TRAIL treatment. In the near future we will explore whether identified resistance genes contribute to TRAIL resistance and increased metastasis in vivo. We anticipate that the identification of novel TRAIL resistance pathways and genes will aid in predicting therapeutic response and provide new targets of therapeutic intervention that may be exploited in combinatorial therapy in breast cancer.

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